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14. ABSTRACT

Resistance to Imatinib mesylate is emerging as a real clinical problem in the management of chronic myelogenous leukemia (CML). In this project, we are exploring the hypothesis that epigenetic silencing associated with promoter DNA methylation mediates resistance in selected cases, and that reversal of silencing by decitabine-induced hypomethylation can be of therapeutic benefit in CML. In progress to date, we have identified samples from patients with CML prior to Imatinib therapy, as well as from patients with established resistance to Imatinib. Bisulfite based analysis identified methylation of p15 and CDH13 in subsets of patients but ruled these genes out as major causes of resistance. In parallel, clinical trials of decitabine have shown activity as single agent and when combined with Imatinib in CML resistant to Imatinib. Analysis of samples from patients on trial showed hypomethylation after therapy. Hypmethylation dynamics suggest that decitabine leads to CML cell death 5-10 days after treatment and suggest that resistance to decitabine is not pharmacologic. These studies are ongoing to clarify the role of methylation in the pathogenesis and therapy of Imatinib resistant CML.

15. SUBJECT TERMS
Chronic myelogenous leukemia, epigenetics, DNA methylation, decitabine, imatinib, clinical trial

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Abstract

Resistance to Imatinib mesylate is emerging as a real clinical problem in the management of chronic myelogenous leukemia (CML). In this project, we are exploring the hypothesis that epigenetic silencing associated with promoter DNA methylation mediates resistance in selected cases, and that reversal of silencing by decitabine-induced hypomethylation can be of therapeutic benefit in CML. In progress to date, we have identified samples from patients with CML prior to Imatinib therapy, as well as from patients with established resistance to Imatinib. Bisulfite based analysis identified methylation of p15 and CDH13 in subsets of patients but ruled these genes out as major causes of resistance. In parallel, clinical trials of decitabine have shown activity as single agent and when combined with Imatinib in CML resistant to Imatinib. Analysis of samples from patients on trial showed hypomethylation after therapy. Hypomethylation dynamics suggest that decitabine leads to CML cell death 5-10 days after treatment and suggest that resistance to decitabine is not pharmacologic. These studies are ongoing to clarify the role of methylation in the pathogenesis and therapy of Imatinib resistant CML.

Subject Terms

Chronic myelogenous leukemia, epigenetics, DNA methylation, decitabine, imatinib, clinical trial

Tasks

Task 1. Determine the methylation profile of candidate tumor-suppressor genes in CML patients treated with Imatinib, months 1-24:

a. Identify in the leukemia database all patients treated with Imatinib at MDACC for whom follow-up of over 1 year is available (month 1)
b. Collect paraffin-embedded pre-treated bone marrow biopsies on all patients (projected 400 patients, 40 cut/month, months 1-10)
c. Extract DNA from paraffin cuts (start month 1 – ongoing until all samples collected, months 1-10)
d. Bisulfite treatment and PCR-based methylation analysis for all the genes (months 2-20)
e. Statistical analysis of the collected data (months 21-22)
f. Validation of the results on prospectively collected samples (months 23-36)

Task 2. Conduct a clinical trial of 5-aza-deoxycytidine followed by Imatinib in patients with CML resistant to, or less likely to respond to Imatinib.

a. Treat initial 6 patients for toxicity analysis of the combination (months 1-2)
b. Enroll patients on the phase II study (projected 3-5/month, months 1-24)
c. Continue subsequent cycles and follow-up of the patients (months 25-36)

Task 3. Determine the methylation and expression status of candidate tumor-suppressor genes after treatment with 5-aza-deoxycytidine and correlate these values with subsequent responses to Imatinib.

a. Collect samples before and after treatment (months 1-24)
b. Analyze samples for methylation (months 13-36)
c. Analyze samples for gene expression (months 13-36)
d. Statistical analysis of the collected data (months 32-36)

Progress on Task 1:

We have obtained samples from 200 patients with CML at various phases with outcome data and have extracted DNA from them (Tasks 1a-1c). A pilot study of multiple genes has identified a panel of genes (P15, LINE, PGRA, PGRB, CDH13, NOR1, NPM2, DPYS, RIL) that are informative for DNA methylation in CML. We are proceeding with bisulfite/pyrosequencing analysis of all these samples (Task 1d) and statistical analysis (Task 1e). At the time of this writing, we have a complete data set on 130 cases, and have run preliminary analyses of the clinical correlations. More in depth analyses are ongoing. Highlights of our findings are:

Mean methylation (%) and frequency of methylation (% of cases with methylation >15)

Gene	P15	CDH13	RIL-1	NOR1	NPM2	PGRA	PGRB	DPYS	AP2E108	ABL75
Mean Methylation (%)	3.7	16.8	23.0	4.7	11.2	11.5	18.8	21.1	40.7	10.9
Frequency of methylation (%)	4.6	40.8	53.1	6.9	15.4	23.8	37.7	48.5	67.7	31.5

Methylation is concordant indicating the presence of hypermethylator phenotypes

Spearman Correlation Tables

CP	P15	CDH13	RIL-1	NOR1	NPM2	PGRA	PGRB	DPYS	AP2E108	ABL75
P15	1.000	0.207	0.146	0.209	0.168	0.369	0.332	0.177	0.037	0.236
	P<0.0001	0.090	0.239	0.088	0.171	0.002	0.006	0.148	0.763	0.055
CDH13	0.207	1.000	0.081	0.053	0.079	0.478	0.212	0.204	0.065	-0.019
	0.090	P<0.0001	0.515	0.670	0.519	P<0.0001	0.083	0.095	0.601	0.877
RIL-1	0.146	0.081	1.000	0.111	0.138	0.059	0.113	0.026	0.233	0.010
	0.239	0.515	P<0.0001	0.371	0.266	0.636	0.365	0.836	0.057	0.935
NOR1	0.209	0.053	0.111	1.000	0.314	0.098	0.241	-0.085	-0.036	0.256
	0.088	0.670	0.371	P<0.0001	0.009	0.426	0.048	0.492	0.773	0.037
NPM2	0.168	0.079	0.138	0.314	1.000	-0.052	0.287	0.259	0.128	0.344
	0.171	0.519	0.266	0.009	P<0.0001	0.674	0.018	0.033	0.299	0.004
PGRA	0.369	0.478	0.059	0.098	-0.052	1.000	0.239	0.299	0.027	0.045
	0.002	P<0.0001	0.636	0.426	0.674	P<0.0001	0.050	0.013	0.825	0.718
PGRB	0.332	0.212	0.113	0.241	0.287	0.239	1.000	0.242	0.137	0.014
	0.006	0.083	0.365	0.048	0.018	0.050	P<0.0001	0.047	0.265	0.910
DPYS	0.177	0.204	0.026	-0.085	0.259	0.299	0.242	1.000	0.051	0.041
	0.148	0.095	0.836	0.492	0.033	0.013	0.047	P<0.0001	0.681	0.744
AP2E108	0.037	0.065	0.233	-0.036	0.128	0.027	0.137	0.051	1.000	-0.071
	0.763	0.601	0.057	0.773	0.299	0.825	0.265	0.681	P<0.0001	0.570
ABL75	0.236	-0.019	0.010	0.256	0.344	0.045	0.014	0.041	-0.071	1.000
	0.055	0.877	0.935	0.037	0.004	0.718	0.910	0.744	0.570	P<0.0001

AP	P15	CDH13	RIL-1	NOR1	NPM2	PGRA	PGRB	DPYS	AP2E108	ABL75

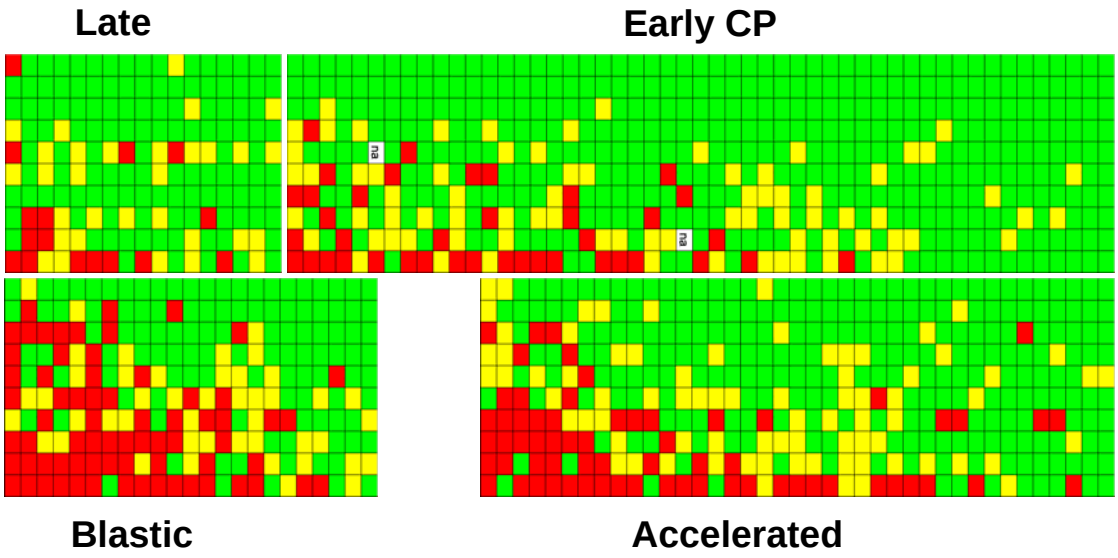
P15	1.000	-0.009	0.233	-0.033	0.259	0.302	0.378	0.343	0.072	0.036
	P<0.0001	0.958	0.154	0.842	0.112	0.062	0.018	0.033	0.665	0.826
CDH13	-0.009	1.000	0.089	-0.098	-0.067	0.416	0.017	0.211	0.216	-0.206
	0.958	P<0.0001	0.592	0.554	0.687	0.008	0.920	0.197	0.187	0.209
RIL-1	0.233	0.089	1.000	0.407	0.192	0.002	0.344	0.198	0.179	0.097
	0.154	0.592	P<0.0001	0.010	0.242	0.991	0.032	0.228	0.275	0.557
NOR1	-0.033	-0.098	0.407	1.000	0.096	0.019	0.270	0.319	0.380	0.070
	0.842	0.554	0.010	P<0.0001	0.560	0.907	0.096	0.048	0.017	0.671
NPM2	0.259	-0.067	0.192	0.096	1.000	-0.174	-0.063	0.324	0.152	0.322
	0.112	0.687	0.242	0.560	P<0.0001	0.290	0.704	0.044	0.355	0.046
PGRA	0.302	0.416	0.002	0.019	-0.174	1.000	0.592	0.333	0.087	0.050
	0.062	0.008	0.991	0.907	0.290	P<0.0001	P<0.0001	0.039	0.599	0.761
PGRB	0.378	0.017	0.344	0.270	-0.063	0.592	1.000	0.283	-0.005	0.180
	0.018	0.920	0.032	0.096	0.704	P<0.0001	P<0.0001	0.081	0.977	0.272
DPYS	0.343	0.211	0.198	0.319	0.324	0.333	0.283	1.000	0.328	0.033
	0.033	0.197	0.228	0.048	0.044	0.039	0.081	P<0.0001	0.042	0.840
AP2E108	0.072	0.216	0.179	0.380	0.152	0.087	-0.005	0.328	1.000	0.036
	0.665	0.187	0.275	0.017	0.355	0.599	0.977	0.042	P<0.0001	0.829
ABL75	0.036	-0.206	0.097	0.070	0.322	0.050	0.180	0.033	0.036	1.000
	0.826	0.209	0.557	0.671	0.046	0.761	0.272	0.840	0.829	P<0.0001

BC	P15	CDH13	RIL-1	NOR1	NPM2	PGRA	PGRB	DPYS	AP2E108	ABL75
P15	1.000	0.065	0.503	0.035	0.544	0.309	-0.044	0.531	0.503	0.267
	P<0.0001	0.768	0.015	0.876	0.007	0.151	0.841	0.009	0.014	0.218
CDH13	0.065	1.000	0.210	0.367	0.538	0.520	-0.061	0.142	0.162	0.122
	0.768	P<0.0001	0.337	0.085	0.008	0.011	0.782	0.517	0.460	0.579
RIL-1	0.503	0.210	1.000	-0.071	0.370	0.331	-0.004	0.411	0.358	0.139
	0.015	0.337	P<0.0001	0.749	0.082	0.123	0.984	0.052	0.094	0.526
NOR1	0.035	0.367	-0.071	1.000	0.248	0.265	-0.211	0.384	0.298	-0.112
	0.876	0.085	0.749	P<0.0001	0.254	0.222	0.335	0.071	0.168	0.612
NPM2	0.544	0.538	0.370	0.248	1.000	0.310	-0.425	0.306	0.333	0.319
	0.007	0.008	0.082	0.254	P<0.0001	0.150	0.044	0.156	0.121	0.138
PGRA	0.309	0.520	0.331	0.265	0.310	1.000	-0.133	0.434	0.379	0.320
	0.151	0.011	0.123	0.222	0.150	P<0.0001	0.545	0.039	0.075	0.136
PGRB	-0.044	-0.061	-0.004	-0.211	-0.425	-0.133	1.000	-0.097	-0.214	0.123
	0.841	0.782	0.984	0.335	0.044	0.545	P<0.0001	0.659	0.327	0.576
DPYS	0.531	0.142	0.411	0.384	0.306	0.434	-0.097	1.000	0.418	0.394
	0.009	0.517	0.052	0.071	0.156	0.039	0.659	P<0.0001	0.047	0.063
AP2E108	0.503	0.162	0.358	0.298	0.333	0.379	-0.214	0.418	1.000	0.154
	0.014	0.460	0.094	0.168	0.121	0.075	0.327	0.047	P<0.0001	0.484
ABL75	0.267	0.122	0.139	-0.112	0.319	0.320	0.123	0.394	0.154	1.000
	0.218	0.579	0.526	0.612	0.138	0.136	0.576	0.063	0.484	P<0.0001

All Pts	P15	CDH13	RIL-1	NOR1	NPM2	PGRA	PGRB	DPYS	AP2E108	ABL75
P15	1.000	0.138	0.259	0.096	0.281	0.328	0.230	0.300	0.168	0.195
	P<0.0001	0.118	0.003	0.276	0.001	0.000	0.009	0.001	0.057	0.026
CDH13	0.138	1.000	0.204	0.208	0.218	0.514	0.278	0.314	0.231	0.009
	0.118	P<0.0001	0.020	0.018	0.013	P<0.0001	0.001	0.000	0.008	0.918
RIL-1	0.259	0.204	1.000	0.262	0.235	0.169	0.270	0.234	0.283	0.076
	0.003	0.020	P<0.0001	0.003	0.007	0.056	0.002	0.008	0.001	0.392

NOR1	0.096 0.276	0.208 0.018	0.262 0.003	1.000 P<0.0001	0.302 0.001	0.223 0.011	0.355 P<0.0001	0.233 0.008	0.195 0.026	0.146 0.098
NPM2	0.281 0.001	0.218 0.013	0.235 0.007	0.302 0.001	1.000 P<0.0001	0.067 0.449	0.125 0.156	0.370 P<0.0001	0.228 0.009	0.362 P<0.0001
PGRA	0.328 0.000	0.514 P<0.0001	0.169 0.056	0.223 0.011	0.067 0.449	1.000 P<0.0001	0.401 P<0.0001	0.387 P<0.0001	0.172 0.051	0.112 0.206
PGRB	0.230 0.009	0.278 0.001	0.270 0.002	0.355 P<0.0001	0.125 0.156	0.401 P<0.0001	1.000 P<0.0001	0.293 0.001	0.128 0.148	0.113 0.201
DPYS	0.300 0.001	0.314 0.000	0.234 0.008	0.233 0.008	0.370 P<0.0001	0.387 P<0.0001	0.293 0.001	1.000 P<0.0001	0.289 0.001	0.162 0.067
AP2E108	0.168 0.057	0.231 0.008	0.283 0.001	0.195 0.026	0.228 0.009	0.172 0.051	0.128 0.148	0.289 0.001	1.000 P<0.0001	0.050 0.575
ABL75	0.195 0.026	0.009 0.918	0.076 0.392	0.146 0.098	0.362 P<0.0001	0.112 0.206	0.113 0.201	0.162 0.067	0.050 0.575	1.000 P<0.0001

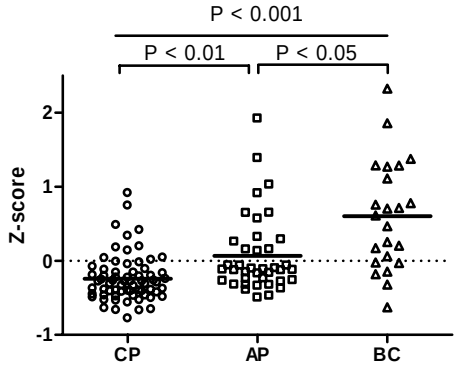
CP: Chronic phase, AP: Accelerated phase, BC: Blast crisis, All: All patients. In each cell, the first value is R and the second is p. Positive correlations in each stage are present, suggesting that a hypermethylator phenotype is present (CIMP). The next figure illustrates this by providing a heat map of all methylation data by stage.



In this map, methylation <15 is in green, methylation 15-30 in yellow and >30 in red.

Methylation increases with increasing stage of CML

Methylation was generally independent of age or gender. We next examined methylation vs. stage of CML. Because methylation is concordant, we derived a methylation index for each case by calculating z-scores and deriving a mean z-score/patient. This was true for all genes (excluding abl) or for a subset of only 3 genes (NPM2, NOR1 and DPYS).



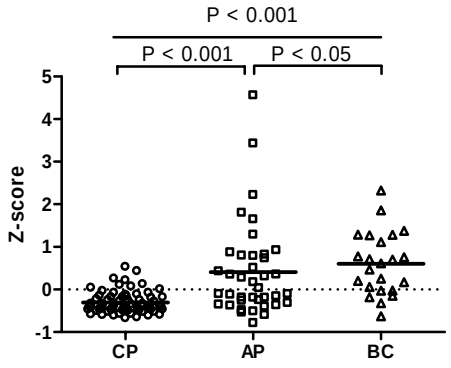
Group 1 genes z-score 1

Kruskal-Wallis test	
P value	P<0.0001

Parameter				Value	Data Set-B
Dunn's Multiple Comparison Test				Difference in rank sum	P value
CP vs AP				-26.60	P < 0.01
CP vs BC				-52.14	P < 0.001
AP vs BC				-25.54	P < 0.05

	CP	AP	BC		CP	AP	BC
Number of values	68	39	23	Median	-0.2880	-0.1190	0.6100

The above figure shows z-scores for all genes in CML cases grouped by stage. The differences between each stage are statistically significant. The differences remain significant when a group of only 3 genes is considered (below).



Group 2 genes z-score 1

Kruskal-Wallis test	
P value	P<0.0001

Parameter				Value	Data Set-B
Dunn's Multiple Comparison Test				Difference in rank sum	P value
CP vs AP				-33.91	P < 0.001
CP vs BC				-51.21	P < 0.001
AP vs BC				-17.30	P > 0.05

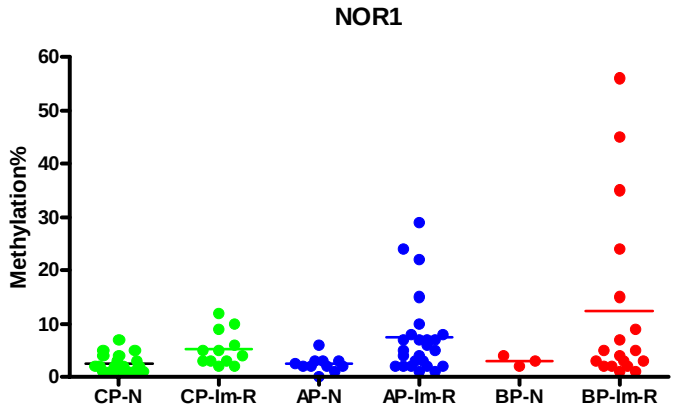
	CP	AP	BC		CP	AP	BC
Number of values	68	39	23	Median	-0.3905	0.03121	0.6100

Methylation increases in imatinib resistant CML

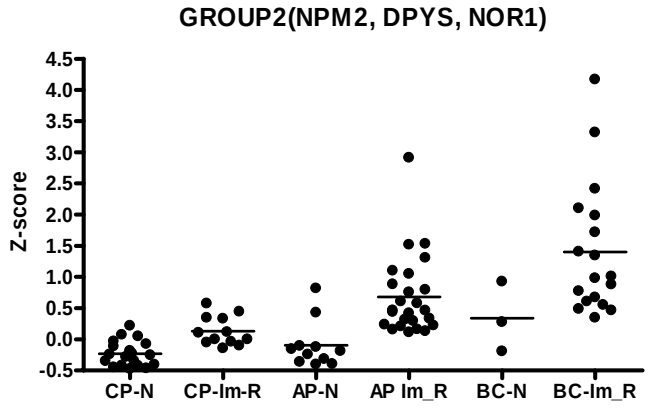
We next examined whether methylation is involved in imatinib resistance in CML, by comparing methylation levels in imatinib naïve and imatinib resistant cases, controlled by stage. The most striking differences were seen for NOR1, which was consistently higher in imatinib resistant cases. The next table has p-values of ttest analysis for imatinib naïve vs. resistant for each stage and each gene.

	CP	AP	BC
P15	0.8745	0.8884	0.8377
CDH13	0.7964	0.7794	0.6918
RIL-1	0.3170	0.3770	0.4686
NOR1	0.0269	0.0032	0.0309
NPM2	0.0007	0.4580	0.1805
PGRA	0.3067	0.0083	0.9575
PGRB	0.1375	0.0366	0.1375
DPYS	0.3662	0.0625	0.3274
AP2E108	0.9873	0.0236	0.9295
ABL75	0.2023	0.0519	0.0120

The next figure shows scatter plots of NOR1 methylation by stage and imatinib resistance.



We also looked at methylation index and imatinib resistance. There we strong associations between the index of NOR1, NPM2 and DPYS with imatinib resistance (see below).



Thus, to summarize progress for this task, we have shown that methylation is frequently abnormal in CML, that it increases with disease stage and that it increases in cases resistant to imatinib, providing rationale for incorporating methylation inhibitors in the treatment of CML.

Progress on tasks 2 and 3

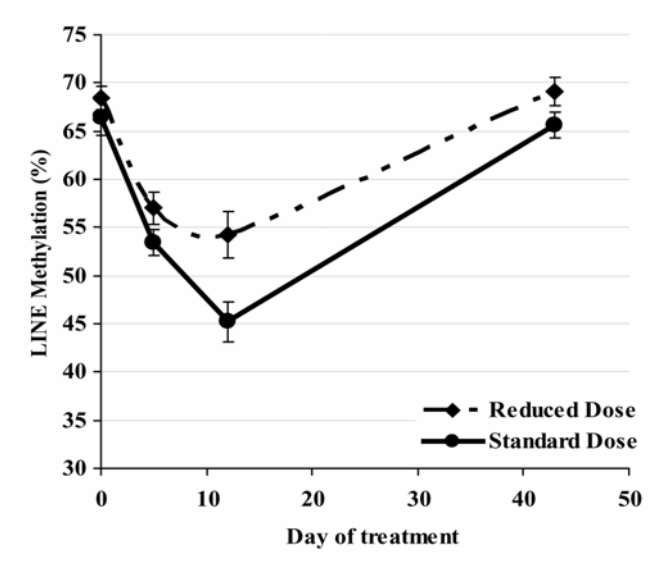
At the outset of the grant, tasks 2 and 3 were modified slightly to include only analysis of samples collected as part of the clinical trials outlined.

A clinical trial of single agent decitabine was performed in Imatinib-resistant CML. This trial included correlative studies funded by this grant and it was recently published[1]. The abstract follows:

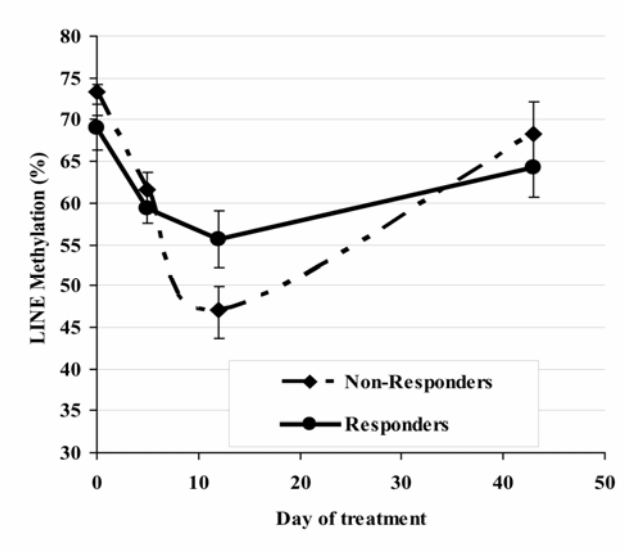
Purpose. To determine the activity of decitabine, a DNA methylation inhibitor, in imatinib refractory or intolerant chronic myelogenous leukemia (CML).
Patients and methods. Thirty five patients were enrolled in this phase II study (12 in chronic phase [CP], 17 in accelerated phase [AP] and 6 in blastic phase [BP]). Decitabine was administered at 15 mg/m^2 IV over one hour daily, 5 days a week for two weeks. DNA methylation was measured using a LINE1 bisulfite/pyrosequencing assay.
Results. Complete hematologic responses were seen in 12 patients (34%) and partial hematologic responses in 7 patients (20%), for an overall hematologic response rate of 54% (83% in CP, 41% in AP and 34% in BP). Major cytogenetic responses were observed in 6 patients (17%), and minor cytogenetic responses were seen in 10 patients (29%) for an overall cytogenetic response rate of 46%. Median response duration was 3.5 months (range 2-13+ months). Myelosuppression was the major side-effect, with neutropenic fever in 28/124 (23%) courses of therapy. LINE1 methylation decreased from 71.3+/-1.4% (mean+/-SEM) to 60.7+/-1.4% after 1 week, 50.9+/-2.4 after 2 weeks and returned to 66.5+/-2.7% at recovery of counts (median, 46 days). LINE1 methylation at the end of week 1 did not correlate with subsequent responses. However, at day 12, the absolute decrease in methylation was 14.5+/-3.0% vs. 26.8+/-2.7% in responders vs. non-responders (p=0.007).
Conclusions. Decitabine induces hypomethylation and has clinical activity in imatinib refractory CML. We hypothesize that the inverse correlation between hypomethylation and response is due to a cell death mechanism of response, whereby resistant cells can withstand more hypomethylation.

Highlights of the correlative studies were:

(1) Methylation decreases in treated patients in a dose-dependent way:



(2) Methylation decreases more in non-responders at 10 days, consistent with a cell death mechanism of action of the drug, whereby non-responders have hypomethylation but do not die.

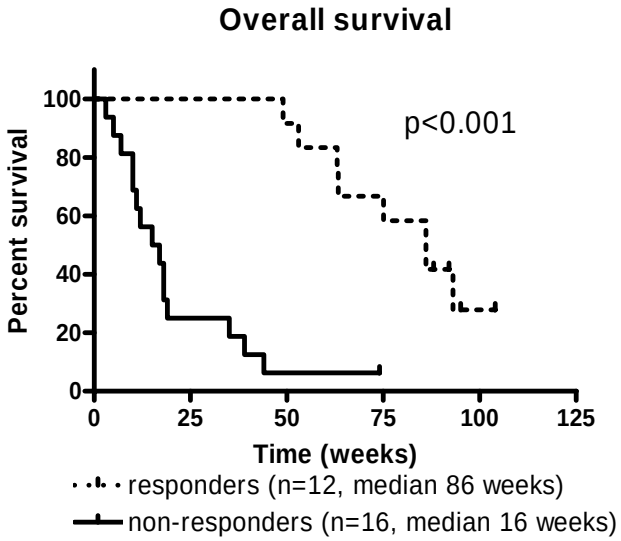


Another study was initiated that combined Imatinib with Decitabine. This study has accrued well. The data are now submitted for publication. A summary of the data follow.

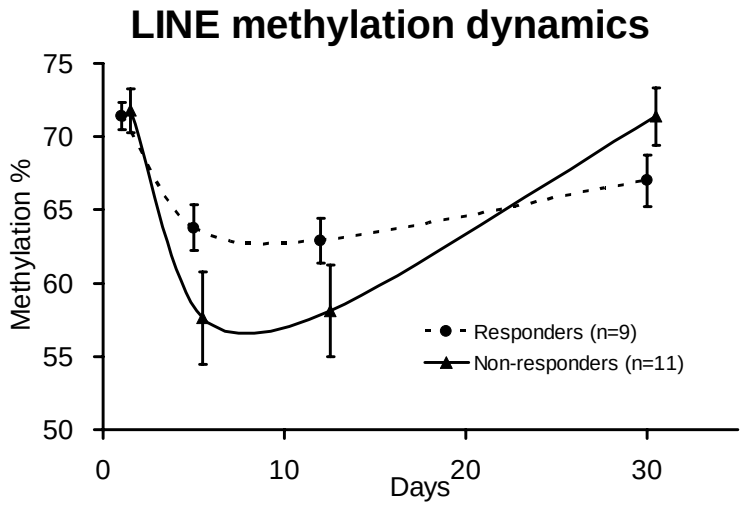
Abstract

Purpose: To determine the activity of decitabine, a DNA methylation inhibitor, in combination with imatinib mesylate (imatinib) in patients with chronic myelogenous leukemia (CML) in accelerated phase (AP) or blastic phase (BP).

Patients and Methods: Patients received decitabine at 15mg/m^2 intravenously daily, 5 days a week for 2 weeks, and imatinib at 600mg orally daily. Global DNA methylation was measured using a LINE bisulfite/pyrosequencing assay.
Results: Twenty-eight patients were enrolled (18 in AP, 10 in BP, and 25 with previous imatinib resistance). A total of 91 cycles (median 2.5 cycles per patient) was administered. Complete hematologic responses, partial hematologic responses, and hematologic improvement were observed in 9 (32%), 1 (4%) and 2 (7%) patients. Major and minor cytogenetic responses were observed in 5 (18%) and 3 (11%) patients. The hematologic response rate was higher in patients without BCR-ABL kinase mutations (1 of 7, 14%) than in patients with mutations (10 of 19, 53%). Median duration of hematologic response was 18 (range 4 to 107+) weeks. Myelosuppression was the major adverse effect, with neutropenic fever in 9 patients (32%). LINE methylation decreased from 71.6%±0.9% (mean ± standard error of the mean) to 60.4%±2.0% on day 5, 60.5%±1.8% on day 12, and returned to 68.8%±1.4% at peripheral blood recovery. Decrease in LINE methylation trended to be greater in non-responders than responders on day 5 and day 12.
Conclusion: Combination therapy of decitabine with imatinib is well tolerated and active in advanced phase CML, particularly in patients without BCR-ABL kinase mutations.



The figure above shows survival in this study by response to the combination.



The figure above shows global methylation dynamics after the combination of DAC+imatinib in responders vs. non-responders.

Key research accomplishments

- Determined that methylation increases with CML progression, and is increased further at Imatinib resistance
- Analyzed samples from patients treated with decitabine in a phase 1 study and showed dose dependent hypomethylation in-vivo
- Analyzed samples from patients with imatinib resistant CML treated with decitabine, confirmed hypomethylation in-vivo, found correlations between degree of methylation at 10 days after therapy and lack of response and found similar hypomethylation at the development of resistance to decitabine in CML, suggesting a non-pharmacologic mechanism of resistance
- Analyzed samples from patients with imatinib resistant CML treated with a combination of imatinib and decitabine and confirmed the above findings

Reportable outcomes

Manuscript published

Issa,J.P., Gharibyan,V., Cortes,J., Jelinek,J., Morris,G., Verstovsek,S., Talpaz,M., Garcia-Manero,G. & Kantarjian,H.M. Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate. J. Clin. Oncol. 23, 3948-3956 (2005).

Manuscript submitted

Yasuhiro Oki, Hagop M. Kantarjian, Vazganush Gharibyan, Dan Jones, Susan O'Brien, Srdan Verstovsek, Jorge Cortes, Charles Koller, Gail M. Morris, Guillermo Garcia-Manero, and Jean-Pierre J. Issa. Phase II study of low-dose decitabine in combination with imatinib mesylate in patients with accelerated or blastic phase of chronic myelogenous leukemia.

Manuscript in preparation:

Jarslav Jelinek, Vazganush Gharibyan, Jorge Cortes, Hagop Kantarjian, Jean-Pierre Issa. Promoter methylation in CML increases with disease progression and imatinib resistance.

Conclusions

Hypermethylation of multiple genes is present in CML, increases with disease progression and is increased at imatinib resistance. Clinical trials of decitabine have shown activity as single agent and when combined with Imatinib in CML resistant to Imatinib. Analysis of samples from patients on trial showed hypomethylation after therapy. Hypomethylation dynamics suggest that decitabine leads to CML cell death 5-10 days after treatment and suggest that resistance to decitabine is not pharmacologic.

"So what": (1) Methylation analysis shows association with Imatinib resistance. Predictive value of such tests will be studied next; (2) The hypomethylating drug decitabine has clinical activity in imatinib resistant CML, and analysis of CML samples after therapy may predict response to this agent.

References

Reference List

1. Issa,J.P., Gharibyan,V., Cortes,J., Jelinek,J., Morris,G., Verstovsek,S., Talpaz,M., Garcia-Manero,G. & Kantarjian,H.M. Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate. *J. Clin. Oncol.* **23**, 3948-3956 (2005).

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